

# Testicular Effects of Phthalate Esters

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The testicular effects produced by di(2-ethylhexyl) phthalate (DEHP) in the rat, characterized by a decrease in the relative organ weight and histological changes in the seminiferous tubules, can also be produced by di-*n*-butyl, di-*n*-pentyl and di-*n*-hexyl phthalates. The corresponding monoesters of these compounds, formed *in vivo* as a result of the action of nonspecific esterases in the intestinal mucosa and other tissues, were equally effective in inducing testicular damage. Phthalate-induced testicular injury was accompanied by a decrease in the zinc content in the gonads and in increased urinary excretion of this element.

Exposure of preparations of rat seminiferous tubule cells in culture to monophthalates capable of producing testicular injury resulted in a dose-related detachment of germinal cells from Sertoli cells in a manner similar to the effect seen in the intact animal. This *in vitro* system may find application in the elucidation of the toxic mechanisms involved in phthalate-induced testicular injury and in screening compounds likely to act in a manner similar to phthalates.

This paper presents a brief review of some of the earlier work on phthalate-induced testicular injury followed by the results of studies conducted at BIBRA.

The first reported finding of phthalate-induced testicular injury in experimental animals was by Shaffer et al. (1) in 1945. The oral administration of di(2-ethylhexyl) phthalate (DEHP) at dietary concentrations of 0.075, 0.75, 1.5 and 5.0% to rats for 90 days resulted in tubular atrophy and testicular degeneration resembling senile changes at the two top dose levels. Subsequently, Harris et al. (2) found occasional incidence of tubular atrophy in rats fed DEHP at 0.5% in the diet for periods of 3 or 24 months.

Other reported instances of phthalate treatment leading to testicular effects in experimental animals were by Calley et al. (3), who found that the daily intraperitoneal administration (250 mg/kg body weight) of DEHP or di(methoxyethyl) phthalate in mice for 6 weeks significantly reduced the relative testicular weight. Furthermore, these two compounds administered IP at half or two-thirds the LD<sub>50</sub> dose levels produced an antifertility effect in male mice (4).

A 90-day feeding study of DEHP in the rat conducted at BIBRA (5) showed that the administration of the compound at 0.2, 1.0 and 2.0% in the diet resulted not only in a dose-related increase in

relative liver weight in the treated animals but also a decrease in the relative testes weight of rats on 1.0 and 2.0% DEHP (Table 1). There was histological evidence of testicular injury, and additionally, "castration" cells in the pituitary at all treatment levels. The histopathological changes in the testes from DEHP-treated animals (Fig. 1) were characterized by a marked reduction in the diameter of seminiferous tubules, the germinal epithelium consisted only of Sertoli cells, spermatogonia and a few spermatocytes, and there was cessation of spermatogenesis. The interstitial tissue and Leydig cells appeared normal. The testicular atrophy produced by DEHP at the 2% dietary level occurred within two weeks of treatment. In the first series of experiments on the ability of phthalate esters to produce testicular injury in the rat, a target organ study of two weeks' duration showed that, whereas diethyl phthalate had no discernible adverse effects on the testes, di-*n*-butyl phthalate (DBP) produced testicular atrophy, possibly more severe than that produced by DEHP.

Studies by Cater et al. (6) on DBP showed that the oral administration of DBP at daily dose levels of 500 and 1000 mg/kg resulted in a significant reduction in the relative testes weight within 6 days and 4 days, respectively (Table 2). The corresponding monoester (MBP), the major urinary metabolite of DBP (7), was found to be even more effective in reducing the testes weight. The histopathology of the testicular lesion produced by DBP or MBP was similar to that following DEHP treatment.

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Table 1. Summary of findings on the testicular effects produced by DEHP in a 90-day feeding study in the rat.

DEHP treatment				Histological findings: incidence of	
Dietary level, % (w/w)	Mean daily intake, mg/kg	No. of rats	Relative testes weight, g/100 g body weight	Testicular injury <sup>a</sup>	"Castration" cells in pituitary
0	0	15	0.60	0	0
0.2	150	15	0.61	4 (+)	1
1.0	750	15	0.41 <sup>b</sup>	12 (++++)	4
2.0	1500	15	0.23 <sup>b</sup>	15 (++++)	9

<sup>a</sup>Severity of testicular damage: (+) slight; (++) moderate; (+++) severe.

<sup>b</sup>Statistical significance  $p < 0.001$ .

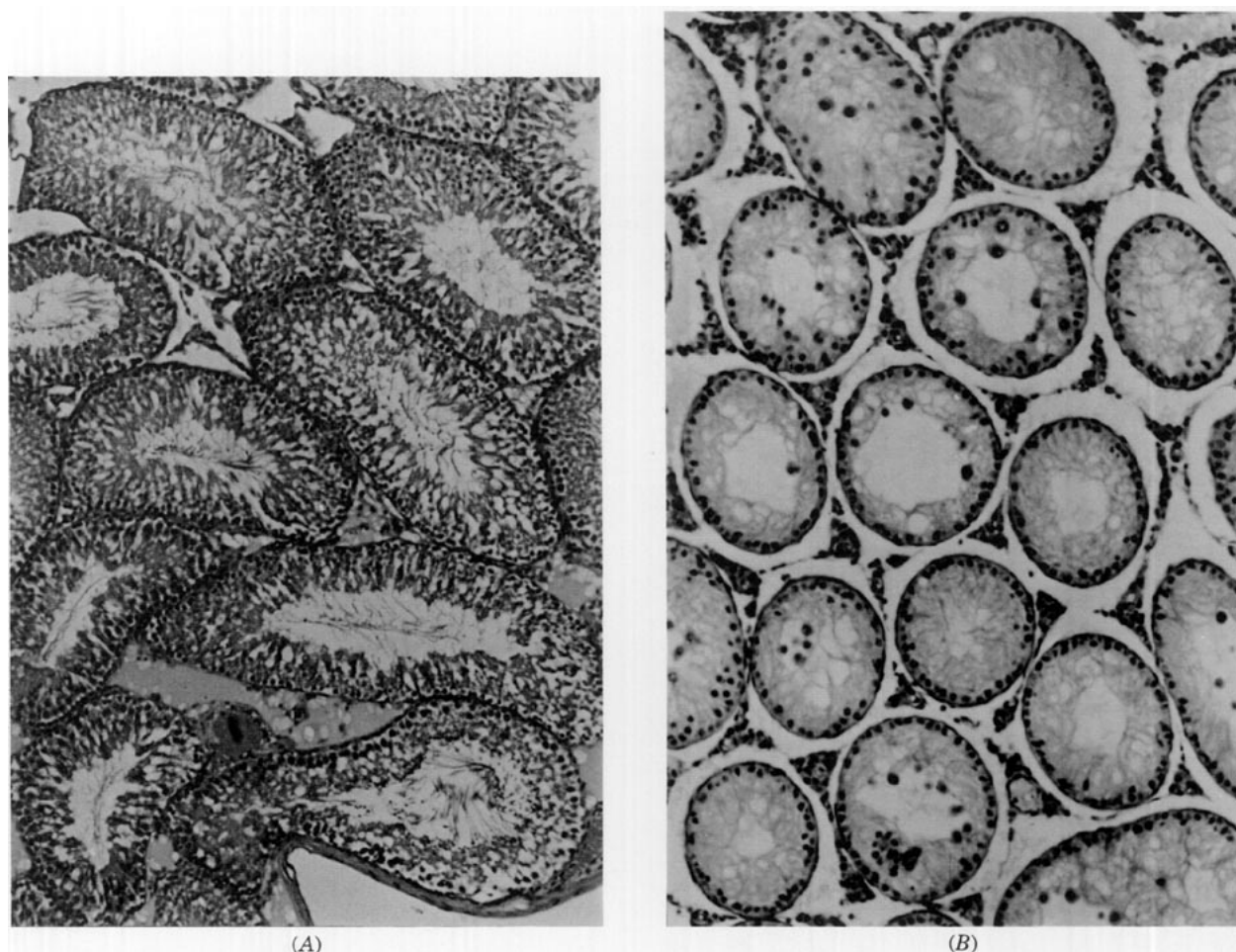


FIGURE 1. Section of testicular tissue from (A) control rat; (B) rat on DEHP (2% in diet) for 90 days. H & E, 160 $\times$ .

Investigations into the likely mechanisms involved showed that DBP-induced testicular injury did not result from the accumulation of metabolites or the formation of covalent adducts in testicular tissue. Metabolic disposition studies following oral administration of  $^{14}\text{C}$ -labeled DBP showed no evidence of accumulation of radioactivity in the gonads. Furthermore, the testicular atrophy did not appear to be mediated by an interference in androgen synthe-

sis or the availability of gonadotrophins. Treatment with testosterone or pregnant mare serum gonadotrophin did not reverse DBP-induced testicular injury (J. B. Gray, unpublished observation).

Measurement of urinary zinc levels by atomic absorption spectrophotometry showed that following DBP treatment there was an increase in the urinary excretion of this element, known to be essential for testicular function (8). Studies us-

Table 2. Effect of DBP or MBP treatment on the relative testes weight in the rat.

Treatment	Dosage, mg/kg/day	Relative testes weight, % of control	
		4 days treatment	6 days treatment
Control (corn oil)		100	100
DBP	500	103	82 <sup>a</sup>
	1000	72 <sup>b</sup>	68 <sup>c</sup>
	2000	66 <sup>c</sup>	57 <sup>c</sup>
MBP	400	78 <sup>b</sup>	64 <sup>c</sup>
	500	66 <sup>c</sup>	53 <sup>c</sup>

<sup>a</sup>Significantly different from control,  $p < 0.05$ .<sup>b</sup>Significantly different from control,  $p < 0.01$ .<sup>c</sup>Significantly different from control,  $p < 0.001$ .Table 3. Effect of DBP treatment on the urinary excretion and testicular content of <sup>65</sup>Zn in the rat.<sup>a</sup>

Treatment time, days (2000 mg/kg body weight)	<sup>65</sup> Zn content, % of control	
	Urine (24 hr collection)	Testes (per 100 mg tissue)
2	125	88
4	175	77
6	125	64

<sup>a</sup>Animals were treated with <sup>65</sup>ZnCl<sub>2</sub> 2 days prior to commencement of DBP treatment.

ing <sup>65</sup>Zn showed that DBP or MBP treatment led to a marked increase in the urinary excretion of radioactivity and a decrease in <sup>65</sup>Zn associated with testicular tissue (Table 3). Furthermore, the loss of testicular zinc in DBP-treated rats was accompanied by a decrease in the activities of two enzymes containing zinc, namely, alcohol dehydrogenase and carbonic anhydrase.

Studies on the testicular effects of monoalkyl phthalates derived from butyl alcohol isomers (9) showed that except for the tertiary butyl ester which was inactive, mono isobutyl and secondary butyl phthalates were as effective as MBP in producing testicular atrophy in the rat. Additional-

ly, only those isomers producing testicular damage were found to alter zinc metabolism by increasing the urinary excretion of zinc and by depleting the concentration of this element in testicular tissues (Table 4). Investigations on the mono-*n*-butyl esters of phthalic acid isomers showed that whereas the *ortho* acid ester damaged testicular tissues in the rat, the *meta* and *para* derivatives were inactive.

Species sensitivity studies provided evidence that the rat, mouse, guinea pig and ferret were susceptible to testicular injury from DEHP and DBP (Table 5). However, the hamster appeared to be resistant to the gonadal effects of these compounds at the dose levels investigated. Furthermore, the corresponding monoesters were also found to be ineffective. A possible explanation for this finding in the hamster will be given later in the text.

As the formation of monoesters is an important metabolic step in the intestinal absorption and gonadal toxicity of oral ingested phthalates *in vitro* hydrolysis studies were conducted on a range of dialkyl phthalates employing small intestinal mucosal preparations from the rat, ferret, baboon and man (10). The results (Table 6) showed that the rate of hydrolysis of a dialkyl phthalate to the corresponding monoester was similar in the experimental

Table 4. Effects of butyl alcohol isomer monophthalates on the testes and zinc-65 metabolism in the rat.<sup>a</sup>

Treatment	Relative testes weight (g/100 body weight)	<sup>65</sup> Zn content		Testicular injury <sup>b</sup>
		Testes, cpm/100mg tissue	Urine, cpm/24-hr specimen	
Control	0.975 ± 0.820	1951 ± 140	5869 ± 952	-
Mono- <i>n</i> -butyl phthalate	0.759 ± 0.127 <sup>e</sup>	1749 ± 138 <sup>e</sup>	6901 ± 221 <sup>b</sup>	+
Mono- <i>sec</i> -butyl phthalate	0.630 ± 0.074 <sup>e</sup>	1712 ± 147 <sup>e</sup>	7583 ± 209 <sup>e</sup>	+
Mono-isobutyl phthalate	0.708 ± 0.081 <sup>e</sup>	1809 ± 132 <sup>c</sup>	9620 ± 549 <sup>e</sup>	+
Mono- <i>tert</i> -butyl phthalate	1.026 ± 0.093	2000 ± 246	6078 ± 202	-

<sup>a</sup>Animals were pretreated with <sup>65</sup>ZnCl<sub>2</sub> 48 hr prior to monophthalate treatment (800 mg/kg/day for 4 days).<sup>b</sup>Testicular injury: - no change from control; + atrophy of seminiferous tubules.<sup>c</sup>Significantly different from control,  $p < 0.05$ .<sup>d</sup>Significantly different from control,  $p < 0.01$ .<sup>e</sup>Significantly different from control,  $p < 0.001$ .

animal species examined, and furthermore, that with increasing length of the alkyl chain the hydrolysis rate decreased. Similar findings were obtained on two human biopsy specimens of small intestine.

Investigations on the testicular effects of a series of di-*n*-alkyl phthalates, ranging from C<sub>1</sub> to C<sub>8</sub>, in the rat (11) showed that whereas the butyl, pentyl and hexyl compounds produced testicular injury, the lower and higher members of the series were inactive (Table 7). Furthermore, in agreement with the earlier finding that phthalate-induced testicular injury was accompanied by an adverse effect on gonadal zinc metabolism, only with those com-

pounds found to act on the male reproductive system in the rat was there as an associated increase in the urinary excretion of zinc and a depletion of this element in the testes. The reason why only three esters of the range of di-*n*-alkyl phthalates investigated produced testicular atrophy is unclear and remains to be elucidated. Intestinal hydrolysis rates does not appear to be the determining factor as treatment with the monoesters of the inactive compounds were also ineffective in producing gonadal injury.

Di-*n*-pentyl phthalate (DPP) was found to be the most potent of the compounds investigated, and testicular injury occurred within 24 hr following a single oral dose in the rat. Histological studies on the early changes produced by DPP (12) showed that within 3 hr of treatment there was evidence of incipient testicular injury characterized by the dissociation of germ cells from the basal membrane of seminiferous tubules. This effect was marked at 6 hr and progressed at 24 hr to the vacuolation of Sertoli cell cytoplasm (Fig. 2). E.M. studies confirmed that the main site of injury in the seminiferous tubules was the Sertoli cells and histochemical succinate dehydrogenase activity in the mitochondria was markedly reduced (Figs. 3 and 4). Further

**Table 5. Testicular effects of phthalate esters in other species of experimental animals.<sup>a</sup>**

Species	Compound	Findings <sup>b</sup>
Mouse	DBP; DEHP	+
Guinea pig	DBP; DEHP	+
Ferret	DEHP	+
Hamster	DBP; DEHP	-

<sup>a</sup>Dosage and duration of phthalate treatment: 2000 mg/kg/day, oral for 10 days.

<sup>b</sup>Findings: + relative testes weight reduced; histological evidence of testicular injury.

**Table 6. *In vitro* hydrolysis of some di-*n*-alkyl phthalates by small intestinal mucosal cell preparations from the rat, ferret, baboon and man.**

Phthalate diester	Amount of product formed, $\mu$ mole/hr/mg intestinal cell protein			
	Rat (4) ( <i>n</i> = 4)	Ferret (3) ( <i>n</i> = 3)	Baboon (4) ( <i>n</i> = 4)	Man <sup>a</sup> ( <i>n</i> = 2)
Dimethyl	1.14 $\pm$ 0.07	0.05 $\pm$ 0.0	6.67 $\pm$ 1.16	111;284
Diethyl	0.648 $\pm$ 0.035	0.053 $\pm$ 0.017	4.33 $\pm$ 0.93	31.2;153
Di- <i>n</i> -butyl	0.590 $\pm$ 0.020	0.063 $\pm$ 0.008	2.19 $\pm$ 0.42	29.1;106
Di- <i>n</i> -octyl	0.219 $\pm$ 0.018	0.083 $\pm$ 0.026	0.190 $\pm$ 0.024	5.8;35.3

<sup>a</sup>Results expressed as nanomoles product formed per mg of total protein.

**Table 7. Testicular effects of a number of di-*n*-alkyl phthalates in the rat.<sup>a</sup>**

Phthalate diester	Dose, g/kg body weight	Testicular injury <sup>b</sup>	Relative testes weight, % of control	<sup>65</sup> Zn content, % of control	
				Urine	Testes
Control (corn oil)	—	—	100 $\pm$ 2.1	100 $\pm$ 3.0	100 $\pm$ 4.5
Dimethyl	1.4	0	111 $\pm$ 2.9	93.2 $\pm$ 10.0	115.2 $\pm$ 4.8
Diethyl	1.6	0	98.6 $\pm$ 3.4	78.8 $\pm$ 4.1	89.1 $\pm$ 5.3
Di- <i>n</i> -propyl	1.8	0	106.7 $\pm$ 2.4	90.7 $\pm$ 2.3	95.5 $\pm$ 5.6
Di- <i>n</i> -butyl	2.0	+	66.9 $\pm$ 6.3 <sup>c</sup>	138 $\pm$ 5.0 <sup>c</sup>	53.1 $\pm$ 2.3 <sup>c</sup>
Di- <i>n</i> -pentyl	2.2	++	56.6 $\pm$ 1.3 <sup>c</sup>	139 $\pm$ 7.1 <sup>c</sup>	38.0 $\pm$ 3.5 <sup>c</sup>
Di- <i>n</i> -hexyl	2.4	+	64.8 $\pm$ 3.0 <sup>c</sup>	133 $\pm$ 2.6 <sup>c</sup>	49.7 $\pm$ 3.1 <sup>c</sup>
Di- <i>n</i> -heptyl	2.6	0	102.7 $\pm$ 2.5	101 $\pm$ 1.1	98.6 $\pm$ 2.0
Di- <i>n</i> -octyl	2.8	0	106.3 $\pm$ 2.3	85.0 $\pm$ 5.7	104.5 $\pm$ 4.9

<sup>a</sup>The compounds were given orally at a dose of 7.2 mmole/kg/day for 4 days to young male animals.

<sup>b</sup>Testicular injury score: (0) no effect; (+) atrophy; (++) severe atrophy.

<sup>c</sup>Significantly different from control, *p* < 0.001.

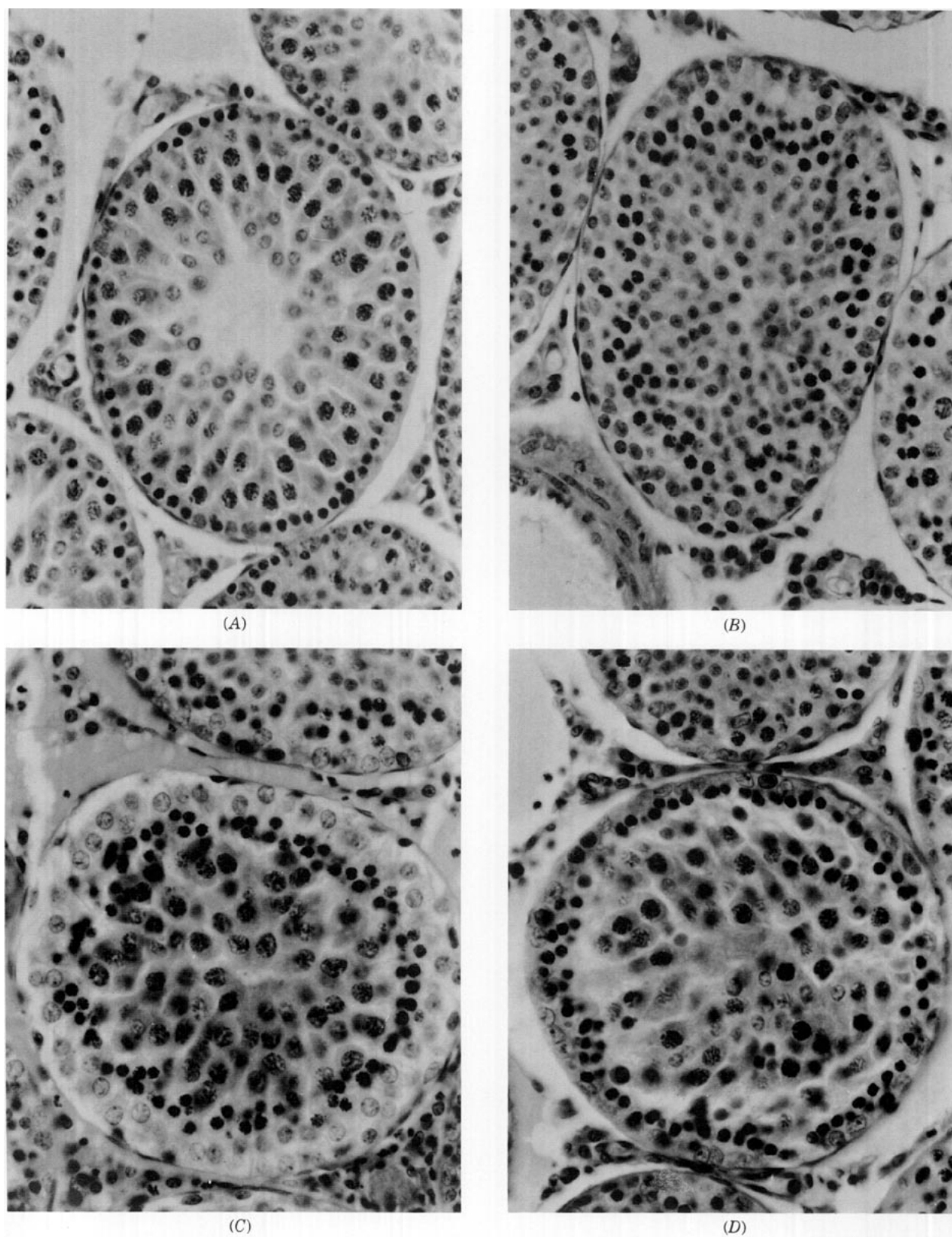


FIGURE 2. Sections of testicular tissues from rats treated with a single oral dose (2000 mg/kg) of di-*n*-pentyl phthalate. (A) at time 0 (control); (B) at 3 hr; (C) at 6 hr; (D) at 24 hr. H & E, 400 $\times$ .

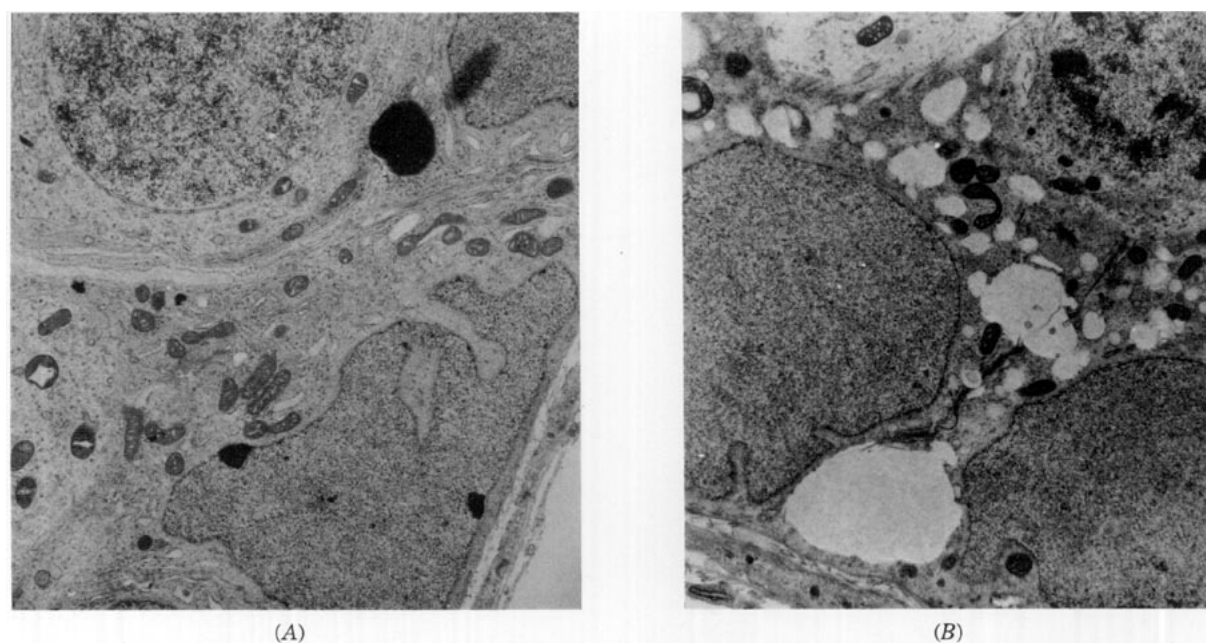


FIGURE 3. Electron micrographs of seminiferous tubule from (A) control and (B) rat 6 hr after a single oral dose of di-*n*-pentyl phthalate (2000 mg/kg).

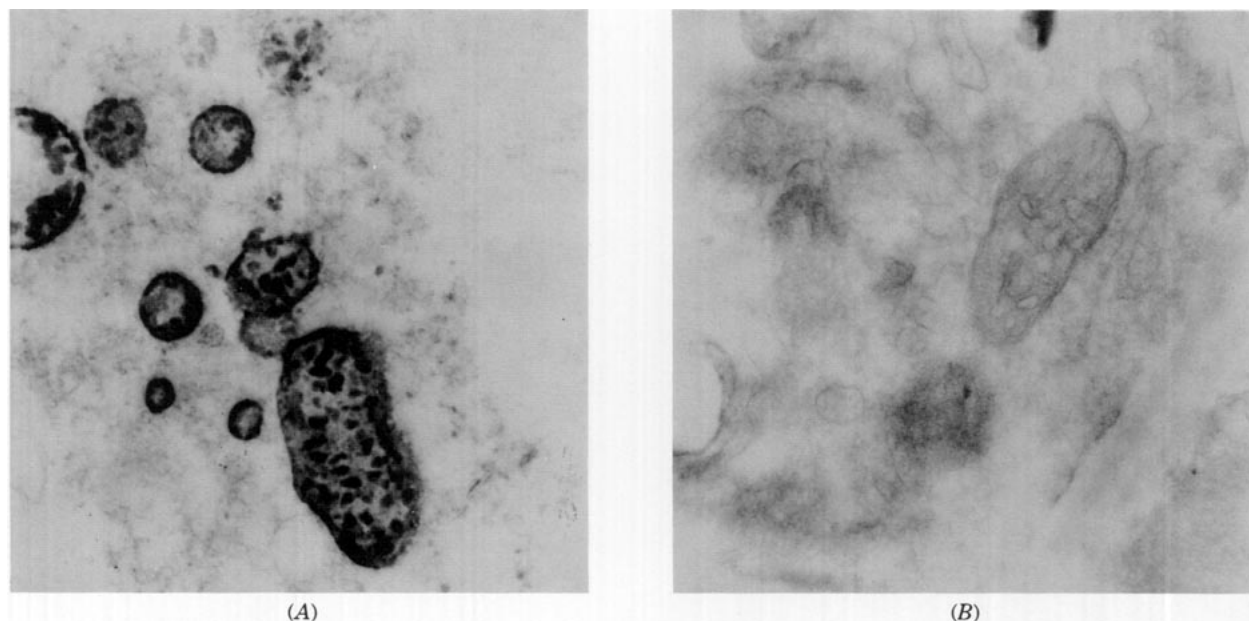


FIGURE 4. Electron micrographs of histochemical succinate dehydrogenase activity in the Sertoli cells of seminiferous tubules from (A) control and (B) rat 6 hr after a single oral dose of di-*n*-pentyl phthalate (2000 mg/kg).

evidence that the Sertoli cells were the main target site was obtained from the finding that phthalate treatment reduced the production of rete testes fluid and of androgen-binding protein (T.B.G Gray, unpublished observation).

The rapidity in the onset of testicular atrophy and the characteristic changes produced in the seminiferous tubules suggested the possibility that tubular cell preparations in culture may respond to phthalates in a manner similar to the intact animal.

**Table 8. Effect of DEHP, MEHP or 2-ethylhexanol on the dissociation of germinal cells from Sertoli cells in cultures of seminiferous tubule cell preparations.**

Treatment	Total number of germinal cells released ( $\times 10^6$ )	
	24 hr exposure	48 hr exposure
Control	5.8 $\pm$ 0.7	4.0 $\pm$ 0.5
DEHP (200 $\mu$ M)	5.8 $\pm$ 0.2	3.9 $\pm$ 0.9
2-Ethylhexanol (200 $\mu$ M)	5.3 $\pm$ 0.7	3.6 $\pm$ 0.6
MEHP (200 $\mu$ M)	18.6 $\pm$ 2.0 <sup>a</sup>	18.3 $\pm$ 3.0 <sup>a</sup>

<sup>a</sup>Significantly different from control,  $p < 0.001$ .

Seminiferous tubules from rat testes were treated with trypsin and collagenase, and the preparations so obtained of Sertoli cells associated with germ cells were maintained in culture with Eagles minimum essential medium. In the first series of experiments, investigations were conducted on the behavior of these cells in culture on exposure to DEHP, mono(ethylhexyl) phthalate (MEHP) and 2-ethylhexanol. The results (Table 8) showed that whereas DEHP and 2-ethylhexanol did not increase above the background value the dissociation of germ cells from Sertoli cells, exposure to MEHP very mark-

edly did so. Thus, MEHP appeared to act directly on seminiferous tubular preparations in culture.

Extending these studies to other monophthalates showed (Table 9) that the compounds causing testicular injury *in vivo* effected a concentration-related increase in the dissociation of germ cells from seminiferous tubule preparations in culture. Conversely, nonactive phthalates were ineffective at the concentrations examined. Interestingly, it has been found that preparations obtained from hamster testes do not respond on exposure to MEHP or MBP. These *in vitro* findings, in agreement with the observed resistance of the hamster to DEHP- or DBP-induced testicular injury suggest that the testicular tissues in the hamster, in contrast to the rat, may have protective mechanisms against phthalate diesters and monoesters.

The close parallel in the response of testicular cell preparations in culture to phthalates and their effects in the intact animal indicates that this *in vitro* system may be of value, not only in studies for the elucidation of mechanisms, but also for screening compounds for testicular effects in experimental animals and in man. Studies directed to these ends are currently in progress.

**Table 9. Effect of monoalkyl phthalates on the dissociation of germinal cells from Sertoli cells in cultures of rat seminiferous tubule preparation on 48 hr contact.**

Compound	Germinal cells detached, % of control				
	10 $\mu$ M	30 $\mu$ M	100	300	1000
Mono(2-ethylhexyl) phthalate	181 <sup>a</sup>	206 <sup>b</sup>	452 <sup>b</sup>		
Mono- <i>n</i> -pentyl phthalate	<sup>c</sup>	179 <sup>a</sup>	223 <sup>b</sup>	257 <sup>b</sup>	474 <sup>b</sup>
Mono- <i>n</i> -butyl phthalate		147 <sup>a</sup>	188 <sup>b</sup>	191 <sup>b</sup>	270 <sup>b</sup>
Mono- <i>tert</i> -butyl phthalate					
Monomethyl phthalate			<sup>c</sup>		
Monoethyl phthalate			<sup>c</sup>		

<sup>a</sup>Significantly different from control,  $p < 0.01$ .

<sup>b</sup>Significantly different from control,  $p < 0.001$ .

<sup>c</sup>No change from control.

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